Acibenzolar-S-methyl against *Botrytis* mold on table grapes *in vitro* and *in vivo*

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ABSTRACT

The objective of this work was to investigate the effect of the resistance inducer Acibenzolar-S-methyl (ASM), against *Botrytis* mold on table grapes *in vitro* and *in vivo*. To assess the effect of ASM on mycelial growth *Botrytis cinerea*, different concentrations (0.125, 0.25, 0.5, 1.0, 2.0 and 3% w:v) were tested. Treatments were set up in triplicate, in a completely randomized experimental design, and replicated twice. Results were expressed in minimum inhibitory concentrations and effective dose per 50% response of mycelial growth. Healthy table grapes (cvs. Italia and Benitaka) were harvested at full ripe to evaluate the ASM 1% effect against gray mold under artificial conditions. Bunches were split into two groups in order to perform two types of experiments (spray or immersion). For both trials, treated bunches were arranged in carton boxes and stored at 2±1 °C, for one month, followed by one week of shelf-life at 22±2 °C. In order to evaluate the ASM effect against gray mold under field conditions, ASM 1% was sprayed on both cultivars one week before harvest. Grapes treated with iprodione 0.2% with three applications during the season were included as a standard chemical control. Bunches were harvested at full ripe, arranged in carton boxes and submitted to a cold storage process as described previously. Results for the *in vitro* experiments showed that the minimum inhibitory concentration of ASM was achieved by 3% and the ED_{50} was 0.04%. Under artificial conditions, for both cultivars, the efficacy of ASM was higher when the grapes were immersed than sprayed. A significant difference was observed for ASM as compared with control. Regarding the effect of ASM against gray mold in the field, the incidence of gray mold was recorded for both cultivars. For 'Italia' and 'Benitaka' grapes, ASM, iprodione and sulfur dioxide pad reduced the incidence of gray mold by 85, 79 and 77%, and by 80.5, 73 and 82%, respectively. As for the physico-chemical berry properties, none of the treatments were significantly different from the control for total soluble solids, titratable acidity and color index. A single ASM treatment applied one week before harvest is effective for controlling gray mold in 'Italia' and 'Benitaka' table grapes.

Key words: Gray mold, table grape, defense elicitors.

INTRODUCTION

The fungus *Botrytis cinerea* Pers. ex Fr.is the second among the world's top 10 pathogens, based on their scientific and economic importance (Dean et al., 2012). Infections by *B. cinerea* can occur before harvest, at the field stage, and they can remain latent until storage, when the pathogen takes advantage for disease development from higher relative humidity and low temperatures, that slow down host defenses. The traditional control of gray mold infection consists of field application of synthetic fungicides during the crop growing cycle. For table grapes, four fungicide applications at the end of flowering, bunch closure, veraison and 3 weeks before harvest are usually carried out (Feliziani et al., 2013). Several families of synthetic fungicides able to control gray mold infections are available (Romanazzi and Feliziani 2014).

However, the use of synthetic fungicides to control gray mold may not be beneficial to our health, so there is a need for alternative control methods. Among these, the use of resistance inducers has the potential for large-scale application. Resistance inducers can increase plant defenses, and, at times, can exploit their antimicrobial properties (Romanazzi et al., 2013). In the field, treatments with resistance inducers can activate the plant defenses and prevent the onset of infections.

Inorganic and organic compounds have been described as systemic acquired resistance inducers. Among

them, acibenzolar-S-methyl is a synthetic molecule whose role as a plant defense activator has been demonstrated in a number of studies. They included studies conducted with apple (Brisset et al., 2000), banana (Furtado et al., 2010; Zhu et al., 2016), strawberry (Mazaro et al., 2008; Romanazzi et al., 2013), melon (Liu et al., 2014) and Sicilian lemon (Panebianco et al., 2014), when applied during pre or postharvest treatments. Acibenzolar-S-methyl, a commercially available plant activator, was reported to induce the plant's defense mechanism(s) by activating the defense gene expression (Pappu et al., 2000; Lawton et al., 1996). Furthermore, the Acibenzolar-S-methyl is used as an elicitor of defense, analog of salicylic acid, against microbial pathogens in agriculture since it offers very low toxicological risk for the environment compared with commonly used pesticides for pest control (Bi et al., 2007; Tomlin 2001). This compound has antifungal, antibacterial, and antiviral activity across a diverse group of plants (Zhu et al., 2016; Amini 2015; Romanazzi et al., 2013; Huang et al., 2012; Pappu et al., 2000; Ishii et al., 1999; Benhamou and Belanger 1998; Görlach et al., 1996).

The resistance induced in fruits by Acibenzolar-S-methyl is related to the accumulation of reactive oxygen species, production of pathogenesis related proteins, and activation of the phenylpropanoid pathway to accumulate lignin, phenolics, and flavonoids (Ge et al., 2012). The phenylpropanoid metabolic pathway influences many crucial disease resistance traits through the synthesis of phenolic compounds, flavonoids, lignin, phytoalexin, and alkaloid, which contribute to plant defense reactions (Jin et al., 2009). The application in melon fruit augmented the content of preformed antifungal compounds, total phenolics and lignin, and caused significant accumulation of lignin, spile and callose in epidermal cells (Bi et al., 2007).

In this context, the objectives of this study were: (i) to investigate the effect of Acibenzolar-S-methyl against *Botrytis* mold on table grape *in vitro* and *in vivo* (ii) to assess the effect of Acibenzolar-S-methyl on maintenance of grape quality profiles (physico-chemical properties).

MATERIAL AND METHODS

Compounds, fungal isolate and grape cultivars

Table grapes (*Vitis vinifera* L., cvs. Italia and Benitaka) were used for artificial inoculation and natural occurring infection. *Botrytis cinerea* was isolated from infected grapes showing typical gray mold symptoms, purified by a single conidia isolation technique and identified based on morphology and molecular methods. For molecular identification, ITS1/ITS4 and beta tubulin primers were used. Isolates were maintained on PDA slants, being stored at 4°C for further use (Youssef and Roberto 2014a). The commercial product Bion® 50% (Acibenzolar-S-methyl 50% silica 10-20% sodium dibutylnaphthalenesulphonate 1-10%, Syngenta Crop Protection, Basel, Switzerland) was used in the experiments. The Rovral® Bayer Crop Science Inc. (*a.i.* iprodione) was included as standard chemical control during the *in vivo* tests. A sulfur dioxide generator pad provided with fast and slow release phases of sodium metabisulfite (Osku S.A. Santiago-Chile) was used during cold storage as a common postharvest treatment. Water control was involved in all experiments as a negative control.

Effect of Acibenzolar-S-methyl on mycelial growth of *B. cinerea*

Acibenzolar-S-methyl was evaluated on mycelial growth of *B. cinerea*. To assess the effect of Acibenzolar-S-methyl on mycelial growth *B. cinerea*, the methods of Youssef et al. (2012) and Youssef and Roberto (2014a) were used. In particular, different concentrations of Acibenzolar-S-methyl 0.125, 0.25, 0.5, 1.0, 2.0, and 3% (w: v) were tested. Treatments were set up in triplicate (randomized experimental design) and the entire experiment was replicated twice. For each concentration, five Petri dishes were utilized as replicates. Radial growth was assessed after six days at 23 ± 1 °C. The results were expressed in minimum inhibitory concentration (MIC) and effective dose per 50% response of mycelial growth calculated using SAS Probit analysis (Sas Institute, Cary, NC, USA) according to Arslan et al. (2009).

Effect of Acibenzolar-S-methyl against gray mold under artificial conditions

Healthy table grape bunches (cvs. Italia and Benitaka) were harvested at full ripe from commercial vineyards located in Paraná (South Brazil). Selected bunches were free of defective or decayed berries, pooled together and the surface sterilized following the methods of Youssef and Roberto (2014b). Bunches

were split into two groups in order to perform two types of experiments (spray or immersion).

During the spray experiment, grape bunches were sprayed with acibenzolar-S-methyl at 1% (w/v) or sterile distilled water (control), until dripping, and left to dry under environmental temperature (25 °C). After 1 hour, bunches were sprayed with a conidial suspension of *B. cinerea* (10^6 conidia mL^{-1}). During immersion experiment, bunches were immersed in Acibenzolar-S-methyl for 5 min (1%, w/v), or in water, and left to dry under environmental temperature (25 °C). Then bunches were inoculated with a conidial suspension of *B. cinerea* (10^6 conidia mL^{-1}).

For both trials, treated bunches were arranged in 23 cm × 16 cm × 9 cm plastic boxes, covered with a 30 cm × 45 cm plastic bag, and stored at 2±1 °C for one month, followed by one week of shelf-life at 22±2 °C and high RH. A completely randomized experimental design including five boxes as replicates (each containing five small bunches and each bunch containing approximately 10–15 berries) were utilized. Both experiments were repeated twice. Results were recorded at the end of cold storage and after one week of shelf-life. Mold incidence was calculated according to the following formula: disease incidence (%) = (number of decayed berries/total number of berries) × 100.

Effect of Acibenzolar-S-methyl against gray mold on the field

The research was carried out in a 5-year-old commercial vineyard of table grapes cvs. Italia and Benitaka, located in Cambira, Paraná State, Brazil. Trials were arranged in a completely randomized design with 5 grapevines (replicates) for each treatment. Acibenzolar-S-methyl at 1% was sprayed one week before harvest using a compressed plastic air sprayer (Brudden Equipments, Brazil). Grapes treated with water were considered negative controls. While grapes treated with iprodione at 0.2% applied three times during the season (after flowering, at pre-bunch closure, at veraison) were included as standard chemical controls, regularly used by farmers in the region.

Bunches were harvested at commercial maturity, showing soluble solids content higher than 14 °Brix, and placed in covered carton boxes (3 boxes per plant, 15 boxes per treatment). Grapes were stored for one month at 2± 1 °C and high RH, followed by one week of shelf-life at 22±2 °C. The entire experiment was replicated twice. The incidence of gray mold was evaluated as described under artificial conditions.

Grape quality parameters (physic-chemical properties)

To assess grape color, TSS (total soluble solids) and TA (titratable acidity), berries were collected from each treatment at the end of cold storage.

Berry color was analyzed using a Minolta CR-10 colorimeter, obtaining the following variables from the equatorial portion of the berries (n = 30 per replicate): L^* (luminosity); C^* (saturation); $h°$ (hue angle) (Cantín et al., 2007). The color index for red grapes (CIRG) was then determined using the following formula: $CIRG = (180 - h°)/(L^* + C^*)$ (Carreño and Martinez 1995).

The chemical analysis of the berries was made according to Youssef and Roberto (2014b). Weight loss (%) was calculated as a percentage of the weight of the fruit at the beginning and at the end of the cold storage period. The difference, as a percentage from the original weight, was calculated (weight loss% = [initial weight − weight at examined date/initial weight] × 100) (Youssef et al., 2015).

Statistical analysis

All data were subjected to an analysis of variance using the Statistica 6.0 software. Mean values for the treatments were compared using the Fisher's protected LSD test and judged at $P \leq 0.05$ level. Percentage data were arcsine transformed to normalize variance.

RESULTS AND DISCUSSION

Effect of Acibenzolar-S-methyl on mycelial growth of *B. cinerea*

The diameter of *B. cinerea* was determined after 6 days incubation at 23±1 °C on PDA amended with different concentrations of Acibenzolar-S-methyl (Figure 1). The minimum inhibition concentration (MIC) of Acibenzolar-S-methyl was achieved by 3%. The percentage of reduction in colony diameter was 63.0, 65.6,

75.0, 80.5, 83.6 and 100% at 0.125, 0.25, 0.5, 1.0, 2.0, and 3.0%, respectively. ED_{50} was 0.04%.

This research used two *in vitro* methods (colony diameter and conidial germination) and no significant difference was observed between both methods (data not shown). ASM could directly inhibit the growth of *Botrytis cinerea in vitro* and the EC_{50} was 3.44 mg mL^{-1} (Muñoz and Moret 2010). Direct effects of ASM on fungal spore germination have only rarely been reported (Koch et al., 2013). Panebianco et al. (2014) reported that spore germination of *Penicillium digitatum* was not affected by ASM at all the tested concentrations, but, in this case, the authors tested ASM concentrations up to 0.5%, which may not have been enough to control *P. digitatum* spore germination.

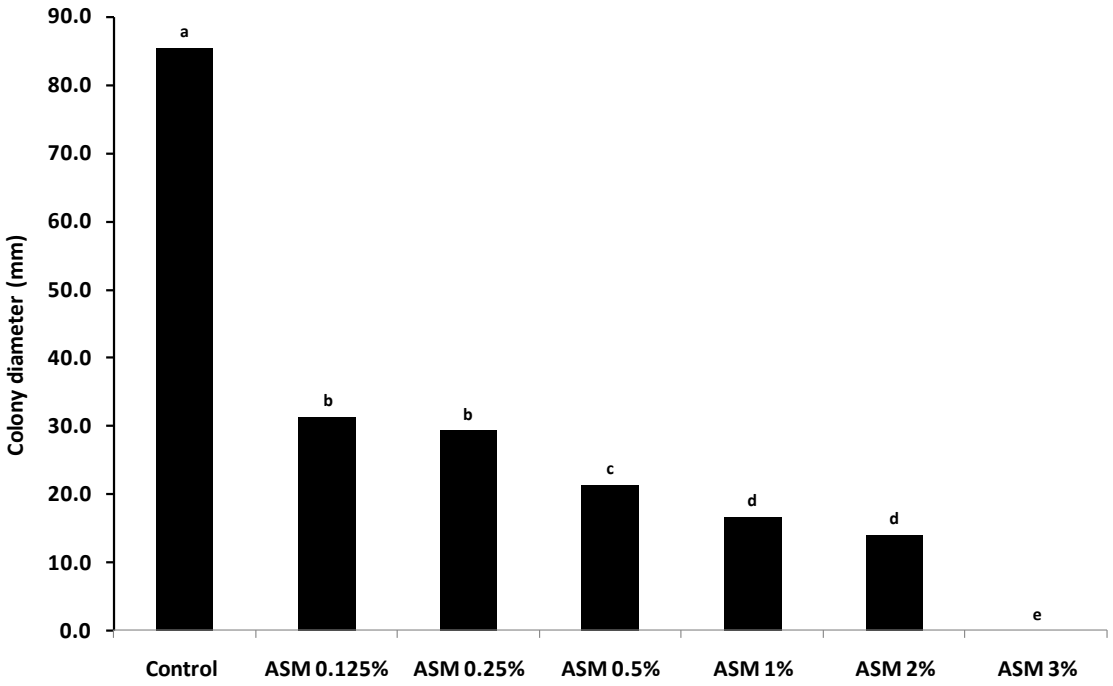

Figure 1. Effect of different concentrations of Acibenzolar-S-methyl (ASM) on the colony diameter reduction of *Botrytis cinerea*. The diameter of *B. cinerea* was determined after 6 days of incubation at 23± 1 ^{o}C on PDA amended with different concentrations (0.125, 0.25, 0.5, 1.0, 2.0, and 3.0%, w/v). Concentrations followed by different letters are statistically different by Fisher's protected least significant difference (LSD) ($P \leq 0.05$).

Effect of Acibenzolar-S-methyl against gray mold under artificial conditions

For 'Italia' and 'Benitaka' table grapes, the percentage of disease incidence was recorded at the end of cold storage and after one week of shelf - life at 22±2 °C. In addition, a significant difference was observed for Acibenzolar-S-methyl as compared with water control in all cases. Particularly, in 'Italia' grapes, at the end of cold storage, Acibenzolar-S-methyl reduced the occurrence of gray mold by 100 and 73%, respectively, as compared with water control in immersion and sprayed applications, respectively (Table 1). For 'Benitaka' grapes, at the end of cold storage, Acibenzolar-S-methyl reduced the occurrence of gray mold by 100 and 70%, respectively, as compared with water control in immersion and sprayed applications, respectively (Table 1). For both cultivars, the same trend was observed after one week of shelf-life. Under artificial conditions, for both 'Italia' and 'Benitaka' grapes, the efficacy of ASM was higher when bunches were immersed rather than sprayed. Furthermore, a significant difference was observed for ASM as compared with water control. Mainly in 'Italia' grapes at the end of cold storage, ASM reduced the occurrence of gray mold as compared with water control under immersion and spray applications by 100 and 73%, respectively, and the same trend was observed for 'Benitaka' grapes.

Artificial inoculation results obtained here confirm the *in vitro* tests of ASM. Those results are in agreement with Amini (2015) who demonstrated that treatments with ASM reduced disease severity of potato *Verticillium* wilt as compared with infected control. The author summarized that the best result for protection of potato plants against *V. dahliae* was conferred by ASM at the dosage of 500 µg a.i.mL^{-1}, which reduced disease severity by 67.8%. In contrast, postharvest treatment of strawberry fruit cv. Camarosa with ASM failed to reduce natural infection by *B. cinerea* at 5 °C (Terry and Joyce 2004). Similar results are described by Panebianco et al. (2014), in which most of the treated citrus fruits ASM did not reduce the

development of green mold (*P. digitatum*), and both disease incidence and severity were similar to those observed in their respective controls.

Table 1. Effect of Acibenzolar-S-methyl solutions - ASM (1%, w/v) on the development of gray mold on 'Italia' and 'Benitaka' table grape bunches artificially inoculated with *Botrytis cinerea* (10^6 conidia mL^{-1}) [1].

'Italia' grape - Disease incidence (%)					
		At the end of cold storage at 2±1 °C		After 7 days of shelf life at 22±2 °C	
Treatment	pH	Immersion	Spray	Immersion	Spray
Water control	6.5	25.0 a[2]	32.0 a	55.0 a	72.0 a
ASM	7.9	00.0 b	8.60 b	3.80 b	20.0 b
'Benitaka' grape - Disease incidence (%)					
	pH	Immersion	Spray	Immersion	Spray
Water control	6.5	20.0 a	23.0 a	58.2 a	77.5 a
ASM	7.9	00.0 b	6.80 b	2.6 b	16.4 b

[1]Grape bunches were cold stored for one month at 2±1°C followed by one week of shelf life at 22 ±2 °C. [2]Treatments followed by different letters are statistically different according to Fisher's protected least significant difference (LSD) ($P \leq 0.05$).

Effect of Acibenzolar-S-methyl against gray mold under field conditions

For 'Italia' and 'Benitaka' table grapes, the percentage of gray mold incidence was recorded after one month of cold storage followed by one week of shelf-life. The three treatments significantly reduced the incidence of gray mold as compared with water control. Also, no significant differences were found between Acibenzolar-S-methyl and iprodione or sulfur dioxide treatments and no phytotoxic effect on the berries or the rachis was observed. For 'Italia' grapes, Acibenzolar-S-methyl, iprodione and sulfur dioxide reduced the incidence of gray mold by 85, 79 and 77%, respectively. For 'Benitaka' grapes, Acibenzolar-S-methyl, iprodione and sulfur dioxide reduced the incidence of gray mold by 80.5, 73 and 82%, respectively (Figure 2A).

The integration of canopy management and fungicide treatments before harvest with the use of sulfur dioxide and cold storage after harvest is the commercial strategy used to implement control gray mold (Droby and Lichter 2004; Gubler et al., 1987). In addition, the selection of plant varieties suited to the growing area and of growing areas that are not suitable for gray mold development is fundamental.

ASM provided significant control of fire blight after leaf infection of apple seedlings and shoot inoculation of apple scions in the greenhouse and after blossom infection of field-grown apple trees (Brisset et al., 2000). ASM has been previously applied as a postharvest treatment against *P. expansum* in peaches (Liu et al., 2005) and pears (Cao et al., 2005), demonstrating that its inhibitory effect is mainly due to the ability to enhance fruit defense responses.

Furthermore, ASM application in 'Maçã', 'Prata', 'Pacovan' and 'Cacau' banana fruits at 0.05%, in postharvest treatment, is effective for controlling antracnosis (*Colletotrichum musae*) in these cultivars (Furtado et al., 2010). According to these authors, the result obtained in this work confirms the ASM effect as a resistance inducer.

Resistance induced by ASM is related to various defense responses, including induction of reactive oxygen species accumulation, activation of phenylpropanoid pathway and production of pathogenesis-related proteins (Ge et al., 2012). In addition, Brisset et al. (2000) discussed the protection of apple from fire blight, which is clearly correlated with the activation of defense mechanisms as indicated by the induction of peroxidasesand-1.3-glucanases. In addition, Benzothiadiazole-mediated treatment enhanced the activities of defense-related enzymes, including chitinase, phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase, increased the content of hydrogen peroxide and total antioxidant capacity, and reduced malondialdehyde content (Zhu et al., 2016).

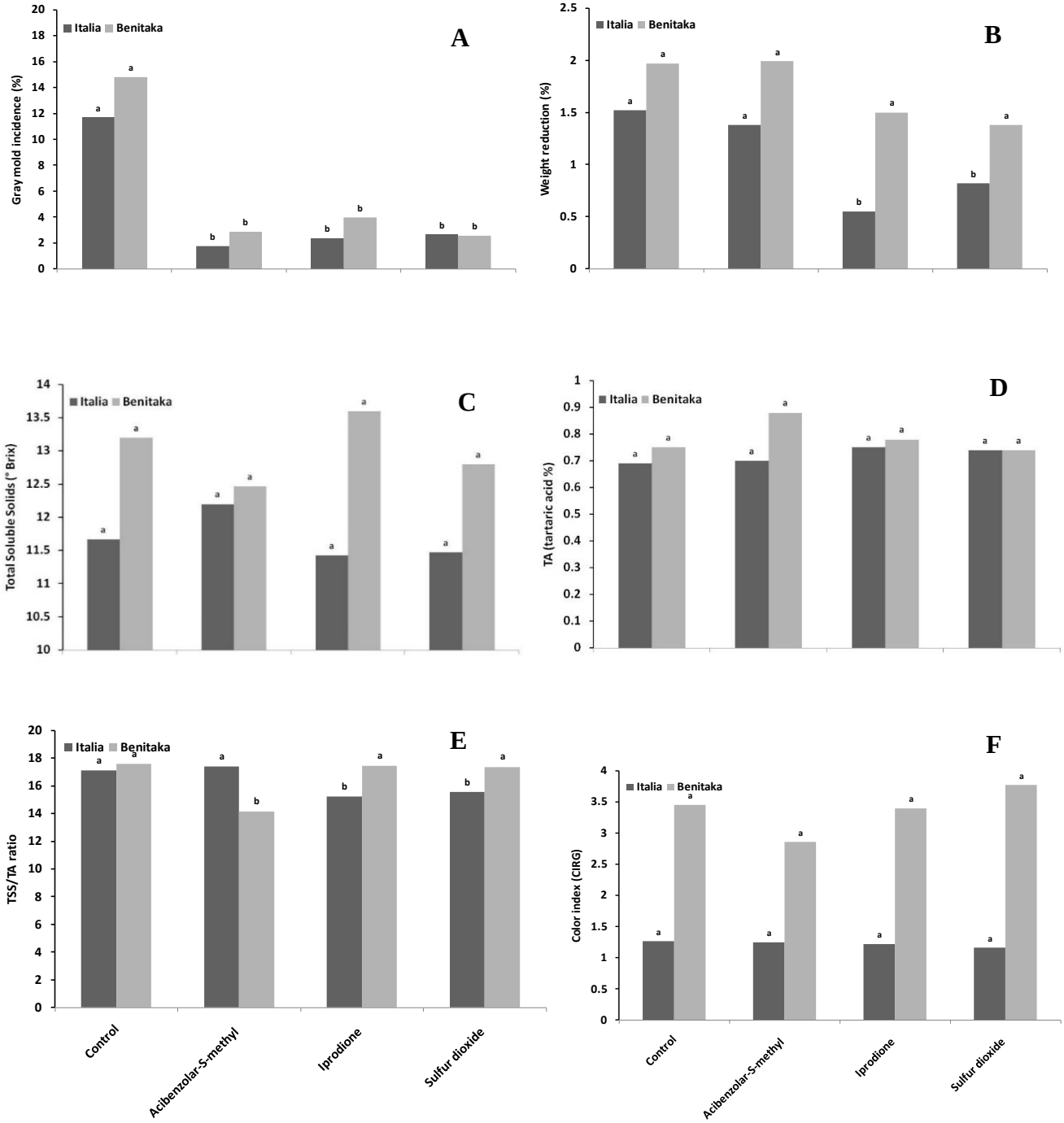

Figure 2. (A) Incidence of gray mold (%) in 'Italia' and 'Benitaka' table grapes after one month of cold storage at 2± 1°C followed by one week of shelf-life at 22±2 °C. (B) Weight reduction (%), (C) total soluble solids - TSS (°Brix), (D) titratable acidity -TA (tartaric acid %), (E) TSS/TA ratio and (F) color index (CIRG) of 'Italia' and 'Benitaka' table grapes at the end of cold storage at 2 ± 1 °C. Acibenzolar-S-methyl was applied at1% one week before harvest. Grapes treated with water, iprodione and sulfur dioxide were used as controls. Columns followed by different letters within each cultivar are statistically different according to Fisher's protected least significant difference test ($P \leq 0.05$).

Grape quality parameters (physico-chemical berry quality properties)

After one month of cold storage, weight reduction percentage was measured for both cultivars. Particularly, in 'Italia' grapes, no statistical differences were found between Acibenzolar-S-methyl treatment as compared with water control, while, iprodione and sulfur dioxide were significantly effective in reducing weight loss by 24 and 30%, respectively as compared with water control. For 'Benitaka' grapes, no statistical differences were found among all treatments as compared with water control (Figure 2B).

After one month of cold storage, TSS, TA, TSS/TA ratio, and color index were evaluated for both cultivars.

Overall, none of the treatments was significantly different from the control for TSS, TA and color index (Figures 2C, D, F). In regards to TSS/TA ratio in 'Italia' grapes, no significant difference was found between Acibenzolar-S-methyl treatment as compared with water control, while, iprodione and sulfur dioxide reduced TSS/TA ratio by 10 and 9%, respectively as compared with water control. For 'Benitaka grapes, Acibenzolar-S-methyl reduced TSS/TA ratio by 19.4% as compared with control, while no differences were found between iprodione and sulfur dioxide pad as compared with water control (Figure 2E).

As previously mentioned by Romanazzi et al. (2012), the influence of treatments on flavor is often ignored since laboratory-scale experiments tend to focus on the effectiveness of a treatment to control decay and do not sufficiently take into consideration the final quality of the produce. The influence of any treatment on flavor is essential for potential commercial application. Concerning the physical and chemical properties, the percentage of weight reduction was measured for both cultivars. Particularly, in 'Italia' grapes, no statistical differences were found between ASM treatment as compared with water control, while, iprodione and sulfur dioxide pad were significantly effective in reducing weight loss by 24 and 30%, respectively.

In addition, no statistical differences were found among all treatments as compared with water control for 'Benitaka' grapes. In this study, a correlation between *in vitro* tests, under artificial infection and natural infection was found for ASM. Overall, none of the treatments was significantly different from the control in TSS, TA and color index. Minor exceptions in case of TSS/TA ratio were found, which were also at an acceptable level. Iprodione and sulfur dioxide reduced TSS/TA ratio by 10 and 9%, respectively. For 'Benitaka' grapes, Acibenzolar-S-methyl reduced TSS/TA ratio by 19.4% as compared with control.

From a practical point of view, our study suggests that only one treatment of ASM, one week before harvest, is effective for controlling gray mold in 'Italia' and 'Benitaka' table grapes. In this study, iprodione was applied three times during the season (after flowering, at pre-bunch closure, at veraison), served as a control. Finally, further biochemical and molecular studies (enzyme activity, gene expression levels and phytoalexin) are needed to clarify the role of ASM as an inducer of systemic acquired resistance against *Botrytis* mold in table grape.

CONCLUSIONS

Only one treatment of Acibenzolar-S-methyl, one week before harvest, is effective for controlling gray mold in 'Italia' and 'Benitaka' table grapes. The acibenzolar-S-methyl treatment does not alter the physico-chemical quality properties of the berry.

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